Shock waves induce activity of human osteoblast-like cells in bioactive scaffolds

Shock Waves Induce Activity of Human Osteoblast-Like Cells in Bioactive Scaffolds

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Background: Bone replacement is frequently needed in periodontal, orthopedic, and maxillofacial diseases. To avoid complications with autografts and allografts, artificial grafts (scaffolds) are candidates for stimulating bone regeneration after colonization with osteoblasts. Moreover, osteoblast activity can be induced by biological or physical stimulation. In this research, extracorporeal shock waves were used to improve the ability of human osteoblasts to colonize scaffolds and to induce their osteogenic properties.

Methods: Osteoblasts, treated with shock waves, were seeded on glass-ceramic macroporous scaffolds. Cells in scaffolds were counted after detachment and examined for calcium nodule formation (Alizarin staining), for differentiation markers (real time polymerase chain reaction), and for scaffold colonization (scanning electron microscope).

Results: Shock waves initially increased both the number and the activity of osteoblasts in the scaffold, but subsequently increased only osteoblast activity. Moreover, shock waves favored scaffold colonization even in the deeper layers.

Conclusions: The calcium deposits and differentiation markers studied have demonstrated that shock waves increase osteoblast migration and penetration into scaffolds.

Clinical Relevance: This study may provide an important starting point for the introduction of shock waves to boost bone formation through osteoblast stimulation in diseases characterized by bone defects.

Key Words: Scaffold, Osteoblasts, Shock waves, BMP, SEM.

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Bone replacements are needed in cases of trauma, neoplasia, and in many periodontal diseases and orthopedic and in maxillofacial surgery.[1–3]

At present, most injuries are not adequately treated because bone defects of critical size cannot be repaired by natural bone growth.[4] Furthermore, because of the increase in mean population age and in surgery for removing tumors, bone regeneration is a clinical need of growing importance.[5] Autografts, allografts, or xenografts can be used as bone substitutes. The high degree of osteoinduction and osteogenesis obtained by autograft makes it the ideal choice. However, it presents some drawbacks, including scarce availability, the need for a second surgical operation, and donor site morbidity.[6–8] Allografts and xenografts, which can overcome these problems, are characterized by poorer bone induction, lower integration rate, by no means of negligible contamination risk, immune rejection, and viral transmission.[9,10] For these reasons, artificial grafts (scaffolds) are interesting and challenging candidates for stimulating bone regeneration and supporting newly formed bone.[1–4,11]

In previous works,[12,13] three-dimensional bioactive glass-ceramic scaffolds were successfully obtained by the sponge impregnation method using a polymeric template. These scaffolds, showing pores in the 100-μm to 500-μm range and trabecular morphology analogous to spongy bone, were highly bioactive as they induced the precipitation of hydroxyapatite on their surfaces. They were also osteoinductive, as evinced by osteoblast proliferation within the scaffold and synthesis of calcium nodules. Because these scaffolds are interesting candidates for bone tissue engineering applications, in this research, human osteoblast-like cells were exposed to shock waves before seeding on the scaffold to increase their osteogenic activity.

The adoption of shock waves to induce bone synthesis was prompted by several considerations. Extracorporeal shock waves were originally introduced in medical therapy to disintegrate calcific deposits within renal, biliary, and salivary tracts.[14,15] More recently, shock waves have also been increasingly applied in various musculoskeletal disorders.[16,17]

Extracorporeal shock wave treatment has also been shown to increase the expression of bone morphogenetic protein (BMP)-2, -3, -4, and -7 in rats with femoral defects.[18]

In vitro studies on human osteoblast-like cells have shown that treatment with shock waves influences cell proliferation enhancing the transmembrane currents and the voltage dependence of Ca-activated and K channels.[19]

Since at the moment little is known about the parameters regarding osteoblast activity induced by shock waves, in this study, we have evaluated alkaline phosphatase (ALP), osteocalcin, type I collagen, BMP-4 and -7, and calcium deposits.

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MATERIALS AND METHODS

Scaffold Preparation

Glass-ceramic macroporous scaffolds were obtained using an organic template (polyurethane sponge) and bioactive glass powders, as previously reported.[12,13] The scaffolds (1 cm^3) were soaked in Tris-buffered simulated body fluid before cell seeding to stimulate the precipitation of the hydroxyapatite layer, known to favor bone formation.

Cell Culture Conditions

Human osteoblast-like cell line, MG-63 (ATCC, Rockville, MD), was grown in minimum essential medium (MEM) medium containing 2 mmol/L L-glutamine, 1% (v/v) antibiotic/antimycotic solution, 1 mmol/L sodium pyruvate, and 10% (v/v) fetal bovine serum in an atmosphere of 5% CO_2 and 95% air at 37°C.

Treatment of Cells With Shock Waves

The shock wave generator used was a piezoelectric device (Piezoson 100, Richard Wolf, Knittlingen, Germany) designed for clinical use in orthopedics and traumatology. The instrument generates focused underwater shock waves at various frequencies (1–4 impulses/s) and intensities (0.05–1.48 mJ/mm^2). For medical use, in orthopedics, shock waves of ~0.01 mJ/mm^2 to 0.6 mJ/mm^2 are applied.[20]

The experimental setup has been previously reported.[21] Briefly, each cell-containing tube was placed vertically. The shock wave unit was kept in contact with the cell-containing tube by means of a water-filled cushion. Common ultrasound gel was used as a contact medium between cushion and tube.

MG-63 cells (10^6/mL) were exposed to shock waves at different energy levels ranging from 0.08 mJ/mm^2 to 0.32 mJ/mm^2. For each energy level, different numbers of impulses were tested (from 50 to 1,000 at 4 impulses/s). MG-63 cells, exposed or not to shock waves, were seeded (10,000 cells/cm^2) in multiwells and used for counting cell numbers and analyzing viability up to 10 days, to identify the shock wave exposure able to increase cell proliferation. After these preliminary experiments, only shock wave treatment corresponding to 0.22 mJ/mm^2 and 100 total impulses was used (named E6 100).

Cell Growth Within Scaffolds

Sterilized scaffolds, pretreated in simulated body fluid for 1 week, were preconditioned for 24 hours in multiwells containing culture medium. After removing preconditioning medium, MG-63 cells, treated with shock waves (0.22 mJ/mm^2 and 100 total impulses) or not (control cells), were seeded (10,000 cells/cm^2) on the scaffolds.

Six, 10, and 20 days after cell seeding, the medium was removed and the scaffolds were used to count the cells that had grown within them and to evaluate cell viability, morphology, presence of calcium deposits, and osteoblast activity parameters. With this aim, the scaffolds were treated with trypsin/ethylenediaminetetraacetic acid (0.25%/0.3%) to harvest the cells present within them.

Cell Count and Viability

Cells were counted in a Burker chamber by using a light microscope (Leitz, Wetzlar, HM-LUX, Germany). To determine viability, plasma membrane integrity was checked microscopically by trypan blue exclusion test (dye concentration 0.8 mg/mL); 400 cells were counted for each sample and results were expressed as percentages of trypan blue-positive cells.

Calcium Deposit Evaluation

The determination of calcium deposits was performed on cells grown within the scaffolds. After trypsinization, fixation in 70% ethanol and washing with Tris-buffered saline, cells were stained with 1% Alizarin red S for 2 minutes, washed with Tris-buffered saline, and observed under light microscope.[22]

Morphology Evaluation by Scanning Electron Microscope

At the different experimental times, scaffolds not treated with trypsin (containing cells), scaffolds not containing cells (negative control), and scaffolds after treatment with trypsin to remove cells were rinsed four times in phosphate-buffered saline and fixed with 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 30 minutes at 4°C. Dehydration was performed with water replacement by a series of graded ethanol solutions with final dehydration in absolute ethanol. Cross-sections of the scaffolds were then gold sputtered for scanning electron microscope (SEM) observation.

Evaluation of Osteoblast Activity Parameters by Real-Time Polymerase Chain Reaction

After 6 days and 20 days, cells detached from scaffolds were examined for osteoblast-activity parameters: ALP, osteocalcin, type I collagen, BMP-7 and -4. Total RNA was extracted using RNeasy Mini Kit (QIAGEN, GmbH, Germany).

Real-time polymerase chain reaction (PCR) was performed using single-stranded cDNA prepared from total RNA (1 μg) with the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). Forward and reverse primers were designed using Beacon Designer software (Bio-Rad, Hercules, CA; Table 1).

Twenty-five microliter of a PCR mixture, containing cDNA template equivalent to 80 ng of total RNA, 5 pmoles each of the forward and reverse primers, and 2× iQ SYBR Green SuperMix (Bio-Rad, Hercules, CA), were amplified using an iCycler PCR (Bio-Rad, Hercules, CA). Each sample of the three different experiments was tested six times and the threshold cycle (Ct) values were the corresponding mean. The fold change was defined as the relative expression compared with that at time 0 (time of seeding cells), calculated as $2^{-\Delta\Delta Ct}$, where $\Delta Ct = Ct_{sample} - Ct_{GAPDH}$ and $\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{time\ 0}$. Data are reported as variation percentages, calculated taking the values of control cells as 100.

Statistical Analysis

All data were expressed as means ± SD of three different experiments. The significance of differences between group means was assessed by variance analysis, followed by the Newman-Keuls test ($p < 0.05$).

TABLE 1. Forward and Reverse Primers for PCR Analysis

Gene Access (Number)	Sequence	Annealing (°C)	Cycles
GAPDH (NM_002046)	FW-5′-GTC GGA GTC AAC GGA TTT GG-3′	52	30
	RV-5′-GGG TGG AAT CAT ATT GGA ACA TG-3′		
ALP (NM_000478)	FW-5′-CTC CCA GTC TCA TCT CCT-3′	58	40
	RV-5′-AAG ACC TCA ACT CCC CTG AA-3′		
Osteocalcin (NM_199173)	FW-5′-GTG ACG AGT TGG CTG ACC-3′	59	35
	RV-5′-CAA GGG GAA GAG GAA AGA AGG-3′		
Type I collagen (NM_000089)	FW-5′-ACA GCC GTC TCA CCT ACA GC-3′	60	45
	RV-5′-GTT TTG TAT TCA ATC ACT GTC TTG CC-3′		
BMP-7 (NM_001719)	FW-5′-GTG GAA CAT GAC AAG GAA T-3′	58	40
	RV-5′-GAA AGA TCA AAC CGG AAC-3′		
BMP-4 (D30751)	FW-5′-CTC GCT CTA TGT GGA CTT C-3′	58	45
	RV-5′-ATG GTT GGT TGA GTT GAG G-3′		

FW, forward primer; RV, reverse primer.

RESULTS

Human osteoblast-like cells, MG-63, were treated with shock waves at different energy levels. For each energy level, the effect on cell proliferation of different numbers of impulses (ranging from 50 to 1,000, 4 impulses/s) was tested (data not shown). On the basis of these preliminary experiments, the energy level and the number of impulses required to stimulate cell proliferation were identified (0.22 mJ/mm^2 per 100 impulses, named E6 100) and adopted in subsequent experiments. Immediately after shock wave exposure, this experimental condition caused a decrease in cell viability (−23%), then a significant increase in cell number, as shown in Figure 1 (panel A), and finally a tendential although not significant increase in the number of cell divisions (panel B). Increase in cell number reached the highest value 10 days after shock wave treatment. The variation percentage calculated for treated cells was 125%, taking the values of control cells as 100. Viability during all experimental times was the same for cells treated with shock waves and control cells and remained about 100% (data not shown). The tendential in-

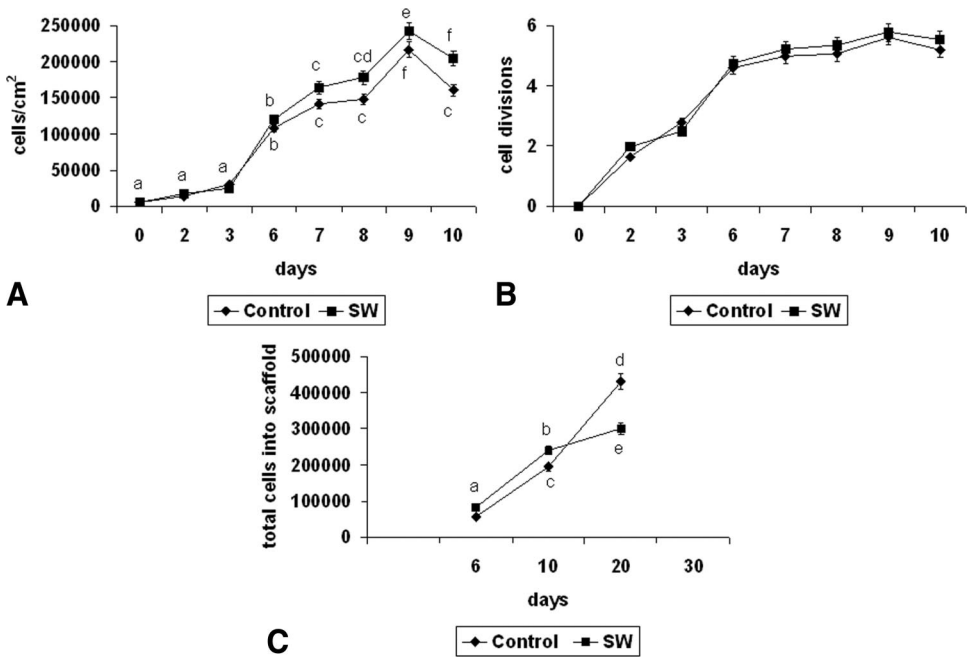

Figure 1. Osteoblast proliferation after treatment with shock waves. *Panel A*: Numbers of osteoblasts were counted after treatment with shock waves at the energy level of 0.22 mJ/mm^2 and 100 impulses (E6 100) at the indicated experimental times. Data are means ± SD of three different experiments. *Panel B*: Numbers of cell divisions were counted as described earlier. *Panel C*: Numbers of osteoblasts, treated with shock waves (E6 100) and seeded on scaffolds, were counted at the indicated experimental times after detachment from scaffold with trypsin. Data are means ± SD of three different experiments. For each panel, means with different letters are significantly different from one another ($p < 0.05$) as determined by analysis of variance followed by post-hoc Newman-Keuls test.

crease in the numbers of new cells generated after treatment with shock waves, compared with those of the control cells, demonstrates the entity of increased colonization of the scaffold by osteoblasts as well as their stimulating effect. Figure 1 also shows the total number of cells present within the scaffold at 6, 10, and 20 days after cell seeding (panel C). In shock wave-treated cells, an increase in cell number was observed at 6 days and 10 days after treatment, whereas a decrease was observed at 20 days.

MG-63 cell spreading and migration within the scaffolds were evaluated by SEM analysis. Different cross-sections of the scaffolds were obtained (up to 5 mm from the surface) to compare the depth of colonization of the shock wave-treated cells with that of the control cells. In both cases, MG-63 cells were observed to attach, spread, and proliferate to a greater degree at 10 days and 20 days than at 6 days. Moreover, SEM analysis showed that at 10 days and 20 days, shock wave-treated cells that penetrated to layers up to 5-mm deeper than control cells, which were only found on the surface. Figures 2 and 3 report SEM micrographs of the surface and of the deepest layer (5 mm) of the scaffolds at 10 days and 20 days, respectively. The cells colonizing the scaffold strongly adhered to its porous structure and appeared to be closely attached to the surface.

To determine the mineralization process, calcium deposits were shown up by staining the cells harvested from the scaffolds with Alizarin red S. Histochemical analysis (Fig. 4) showed that after 10 days, a few Alizarin-positive areas had developed only in scaffolds colonized by shock wave-treated cells; at 20 days, calcium deposits were observed in both control and shock wave-treated cells, but they were more

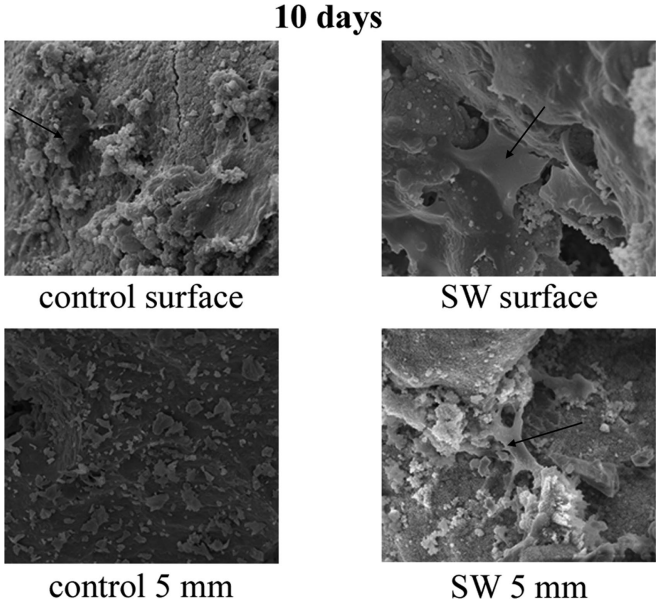

Figure 2. Osteoblast spreading and migration onto the scaffolds evaluated by SEM analysis at 10 days. SEM micrographs of the surface and of deepest layer (5 mm) of the scaffolds show the osteoblasts treated (E6 100) or not treated with shock waves at 10 days after seeding on the scaffolds. Arrows indicate cells.

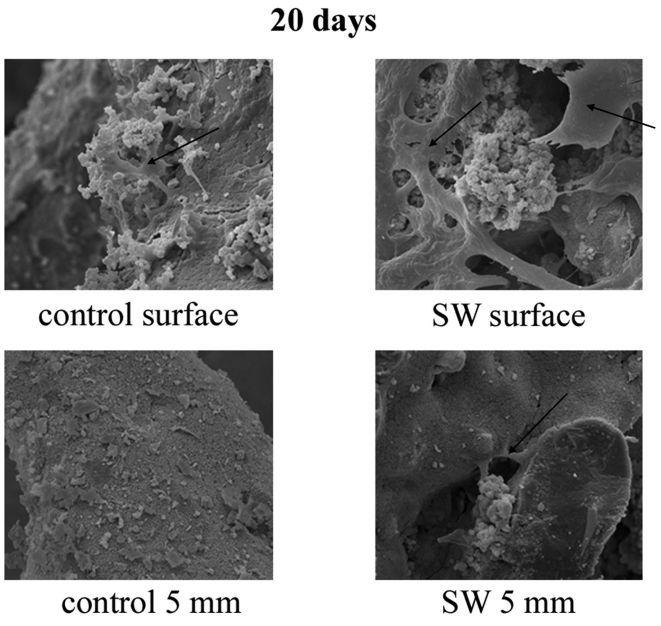

Figure 3. Osteoblast spreading and migration onto the scaffolds evaluated by SEM analysis at 20 days. SEM micrographs of the surface and of deepest layer (5 mm) of the scaffolds show the osteoblasts treated (E6 100) or not treated with shock waves at 20 days after seeding on the scaffolds. Arrows indicate cells.

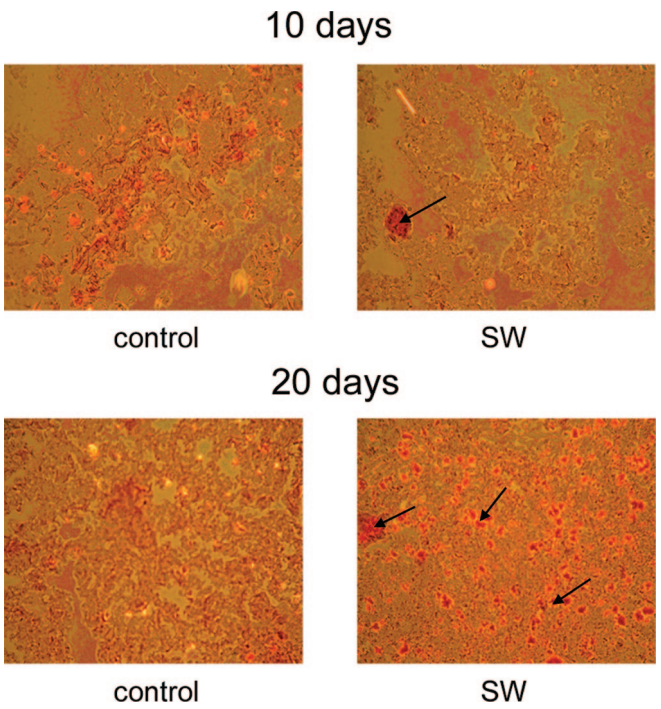

Figure 4. Calcium deposits evidenced with Alizarin S staining. Osteoblasts harvested from the scaffolds were stained with Alizarin red S and observed at light microscope. The cells treated (E6 100) or not treated with shock waves were harvested at 10 days and 20 days after seeding on the scaffolds. Arrows indicate calcium deposits.

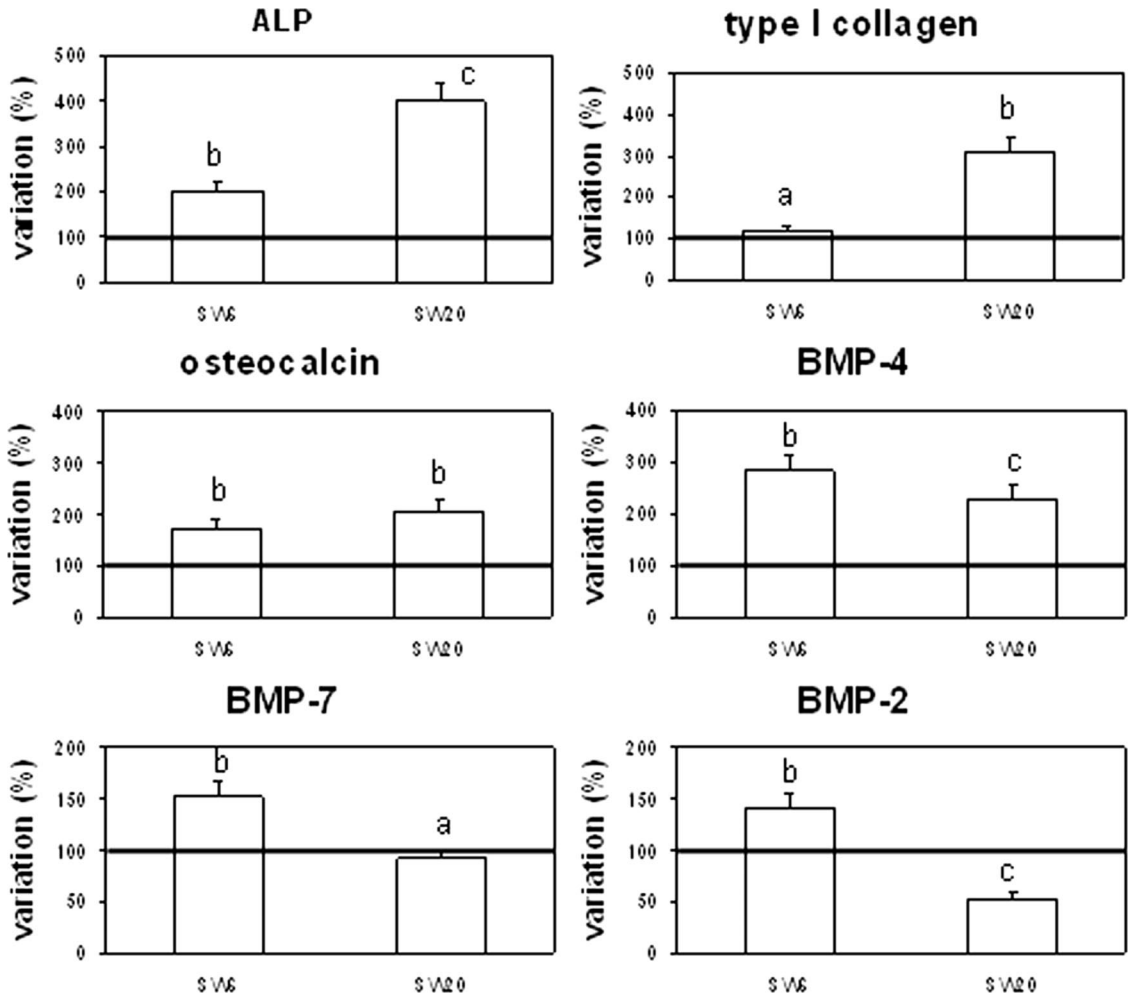

Figure 5. Alkaline phosphatase (ALP), osteocalcin, type I collagen, bone morphogenetic protein (BMP)-4, -7, and -2 mRNA content. mRNA content, at 6 days and 20 days after seeding cells on scaffolds, was evaluated by real-time PCR and the values of osteoblasts treated with shock waves (E6 100) were referred to those of control cells taken as 100% (*black line*). Data are means ± SD of three different experiments. For each panel, means with different letters are significantly different from one another ($p < 0.05$) as determined by analysis of variance followed by post-hoc Newman-Keuls test. The control values are indicated as a.

frequent and larger in scaffolds containing shock wave-treated cells.

Markers of osteoblast activity were also examined. ALP, type I collagen, BMP-7, -4, and osteocalcin mRNA were evaluated by real-time PCR at 6 days and 20 days after shock wave exposure. Figure 5 shows that all the parameters examined were higher in shock wave-treated cells than in control cells, except for type I collagen at 6 days, BMP-7, and -2 at 20 days, when the values were 120%, 90%, and 53%, respectively, with control cell values taken as 100. The percentage values of increased gene expressions in treated cells were at 6 days, 200% for ALP, 173% for osteocalcin, 283% for BMP-4, 152% for BMP-7, and 141% for BMP-2; at 20 days, 422% for ALP, 312% for type I collagen, 207% for osteocalcin, and 230% for BMP-4.

DISCUSSION

Current opinion holds that significant improvements in bone regeneration will be obtained only by using new technologies based on tissue engineering supported by biochemical or biophysical stimulation. This science requires three-dimensional scaffolds able to mimic bone and to be colonized by osteoblast-like cells and their products. Among possible forms of biophysical stimulation, shock waves have recently been applied in a broad range of musculoskeletal pathologies,[16,17] even though some aspects of the mechanisms involved are still unclear. In this research, it has been found that treating human osteoblast-like cells, MG-63, with shock waves generated by a piezoelectric apparatus produces an increase in the number of osteoblasts and their degree of penetration into the scaffold. Regarding the increase in osteoblast number, the effect was different at various experimental times: at the earlier times (6 days and 10 days after shock wave exposure), numbers of shock wave-treated cells present within the scaffold were higher than those of control cells, whereas at the last experimental time (20 days), the numbers were lower than in the controls. Conversely, it has been shown that there were more

and larger calcium deposits present in scaffolds colonized by shock wave-treated cells than those produced by control cells. In the light of these observations, we supposed that shock wave-treated cells at 20 days might proliferate less but function more actively. To measure the parameters induced by shock waves resulting in an increased osteogenic activity of MG-63 cells, some markers have been studied. It has emerged that MG-63 cells exposed to shock waves expressed more ALP, osteocalcin, type I collagen, and all BMP-4, -7, and -2. ALP, type I collagen, and osteocalcin showed the highest increase after 20 days, whereas BMP-4, -7, and -2 showed the highest increase after 6 days with a decrease after 20 days. It is known that ALP expression, which is an early marker of osteoblast differentiation, could be increased by BMP-4.[23,24]

BMPs enable skeletal tissue formation during embryogenesis, growth, adulthood, and healing. BMPs (BMP-2, -4, and -7) are the only growth and differentiation factors, which can singly induce de novo bone formation both in vitro and at heterotopic sites in vivo.[25] After a fracture, BMPs apparently diffuse from reabsorbing bone matrix and activate osteoprogenitor cells which, in turn, produce more BMPs. The temporal and spatial distribution of the BMPs during fracture healing have been moderately well characterized as a complex, interactive, and site-specific process.[26–28]

The observations described earlier suggest that shock waves initially induce an increase in cell number and osteogenic activity, whereas induction of osteogenic activity prevails later.

This research has shown that the use of physical stimulus, such as shock waves, induces osteoblast activity producing the same effect as using biological molecules, such as BMP-2, an osteoinductive growth factor able to determine osteoblast differentiation by increasing calcium deposits and accelerating the healing process when implanted in a bone defect. Our previous article[12] described how in scaffolds colonized with human osteoblast-like cells treated with BMP-2, more and larger calcium deposits were produced than in the controls, suggesting that the higher degree of mineralization ability of the cells, although there was only a small number of them in the scaffold, was attributable to the BMP-2. In the same way, in SEM analysis, osteoblast-like cells were observed to attach, spread, proliferate, and form mineralized nodules when cultured on bioactive scaffolds to a greater extent in BMP-2–treated cells than in controls.[12] Therefore, shock waves treatment may be assumed to provide a good opportunity to stimulate osteoblast activity, preferable to the use of chemical substances.

It should be noted that the importance of the use of shock waves lies in their ability to stimulate scaffold colonization and migration: shock wave-treated cells have been found to penetrate further into scaffolds, whereas untreated cells remain on the surface.

These encouraging results have led us to conclude that this study may well provide an important point of departure in the introduction of shock waves to enhance bone healing through osteoblast activity in bioactive glass-ceramic scaffolds.

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